ANALYSIS OF Bcl I AND Xba I POLYMORPHISM IN FACTOR VIII GENE TO DETECT CARRIERS OF HAEMOPHILIA A IN ANDHRA PRADESH.

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ABSTRACT

The efficacy of two intragenic polymorphic markers of factor VIII gene has been examined in Andhra Pradesh population with a view to confirm/revise the strategy for carrier detection that would be precise and economical. The haemophilia A carrier was detected using Bcl I and Xba I polymorphic sites in intron 18 and 22 respectively. The cumulative efficiency of these two sites for detection of carriers is 100% since all 15 families tested were informative for one of these polymorphisms, thus confirming their usefulness for factor VIII gene mutations found in Andhra Pradesh.

KEY WORDS

Haemophilia A, PCR, Bcl I and Xba I polymorphisms and carrier detection

INTRODUCTION

Due to high heterogeneity of mutations causing haemophilia A and considering the size and structural complexity of factor VIII gene, direct diagnosis of molecular defect causing haemophilia A is difficult. Most of the disease causing mutations are sporadic and new mutations are bound to occur. However, polymorphisms have been used to track mutant gene and hence follow it in a pedigree. To be of diagnostic use, a polymorphism must differentiate the two X-chromosomes of a female carrier. In haemophilia gene, the most useful polymorphisms are bi-allelic. They are formed by a single nucleotide substitution that either creates or deletes a restriction enzyme cleavage site in the gene. If there is a variation in normal polymorphic site, the cutting site of restriction enzyme is altered, and hence these fragments will vary in length not only from one individual to another, but also between the two X-chromosomes of a female. This can be used to identify the gene(allele) that carries the mutation(1). There are two other useful multiallelic polymorphisms in the CA repeats of FVIII gene. One is in intron 13 and the other in intron 22. In addition, there are two extragenic

polymorphisms adjacent to FVIII gene that carry a risk of recombination (hence at risk of misdiagnosis up to 5%). The heterozygosity of a woman for any polymorphism decides its usefulness in tracking X-chromosomes carrying an affected gene. Population based variations have given a lot of scope for further study in other ethnic groups in order to assess their usefulness.

Bcl I and Xba I markers have been found to be useful in migrant Indians and many other populations (2). Shetty *et al* (3) have reported of the usefulness of these markers in some of the families from the sub continent. In a more recent study Roy *et al* (4) reported two new markers Hind III and intron 22 dinucleotide repeats in Indian population.

In India with its large population, the estimated number of haemophilia A patients is over 50,000. This alone prompts one to compile data on the nature of mutations occurring in our population and also documenting useful polymorphic sites associated with this gene. We have attempted PCR based analysis of two polymorphic sites on factor VIII gene in haemophilia A families in Andhra Pradesh.

MATERIALS AND METHODS

Blood samples were collected from 15 known haemophilia A families (total subjects 43 comprising of 23 females and 20 males). The patient's factor

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VIII levels ranged from >1 to 12 units/dl. DNA was extracted from whole blood of normal subjects and patients of haemophilia A and their relatives using standard protocol (5).

PCR conditions for Bcl I polymorphism analysis:

250 ng of genomic DNA was added to a PCR mix containing 200 μ M dNTP's, 10 x PCR Taq buffer (10mM Tris (pH 8.3), 50mM KCl, 15 mM $MgCl_2$ and 0.01% Gelatin), 25mM $MgCl_2$, 1.5 units of Taq polymerase and 10 pmol each of oligonucleotide primers (8.1 and 8.2, Table 1). The solution was made upto 25 μ l with distilled water. Initial denaturation temperature was setup at 95°C for 5 minutes after which 29 cycles of PCR was performed each cycle consisting denaturation for one minute at 90°C annealing for 1 minute at 62°C and extension step at 72°C for 1 minute, final elongation step was for 10 minutes. After confirming amplification, the remaining PCR product was subjected to 3 units of Bcl I restriction enzyme digestion at 55°C over night. The digested product was then electrophoresed on polyacrylamide gel as described by Kogan *et al* (6). The amplified fragment size from mutant chromosome was 142 bp (due to abolition of this site).

PCR conditions for Xba I polymorphism analysis:

250 ng of genomic DNA was added to an aliquot containing 10 x PCR Taq buffer (10mM Tris (pH 8.3), 50mM KCl, 15 mM $MgCl_2$ and 0.01% Gelatin) 200 μ M dNTP's, 1.5 units of Taq polymerase and 10 pmol each of oligonucleotide primers (7.1 and 7.10, Table-1). After an initial denaturation of 95°C for 5 minutes, PCR amplification was carried out for 34 cycles each consisting of the following: denaturation for 30 seconds at 95°C, annealing at 62°C for 30 seconds and extension step at 72°C for 30 seconds. This was followed by an extension step at 67°C for 5 minutes. Amplified product is 96 bp. Restriction enzyme digestion was carried out by the addition of 3 units of Xba I restriction enzyme and incubating at 37°C over night. Digested samples were run on 12% polyacrylamide gel at 70V for one hour followed by ethidium bromide staining. Normal samples give 96bp band and carriers give 96,68 and 28 bp bands.

RESULTS AND DISCUSSION

Figure 1 shows analysis and carrier

identification with Bcl I polymorphism. Amplified samples after digestion with Bcl I are displayed on polyacrylamide gel stained with ethidium bromide. The 142 bp Bcl I fragment is cleaved into 99 and 43 in heterozygotes (lane 3) whereas in carrier female (who is heterozygous for this polymorphism) 142, 99, and 43 bp (lane 2). Lane 4 represent patients sample (142 bp). It is evident that Bcl I polymorphism is useful in tracking chromosome in this family.

Figure 2 illustrates the informativeness of Xba I polymorphism. DNA samples from this family were amplified using primers 7.1 and 7.10 (Table 1). The amplified 96 bp fragment corresponds to regions of the factor VIII gene as well as a homologous region in the human genome elsewhere (7). When digested with Xba I the association of the disease with Xba I in this family is confirmed by the presence of 68 bp and 28 bp products. Table 2 represents the details of family studies, range of FVIII levels and informativeness for the polymorphic markers used. Out of 15 families studied 13 families are severe to moderately severe (< 2 to 2 - 10 units/dl) and 2 families are mild (>10 -30 units/dl of F VIII).

10 families out of 15 tested were informative for Bcl I polymorphism (intron 18) which is almost 67% whereas in 33% of families Xba I for intron 22 was informative. In all the 15 haemophilia A families studied either one of these two polymorphic markers were informative i.e., heterozygous in the female. Surprisingly in the 10 families which are informative for Bcl I polymorphic site, most of them are severe to moderately severe cases.

Though there is a marked variation in the polymorphisms associated with this disease among different ethnic groups, Bcl I and Xba I are found to be two useful ones for haemophilia A diagnosis (2,8-11). This study was conducted to confirm the usefulness of these two intragenic markers in A.P population. In all the 15 families both the polymorphisms were tested. We were able to give carrier status in the female siblings. However, Bcl I is the predominant marker where the heterozygosity rate in the females was 67%. Xba I proved to be a good combination as it was informative in the rest of the families thereby giving a 100% carrier detection rate. This study underlines the importance of screening for Bcl I and Xba I polymorphic sites in the Indian

subcontinent for routine diagnosis and genetic counseling. Although we have not tested as many polymorphisms reported by others (4) and also the number of the families tested are small, the importance of testing the above two polymorphisms can not be minimized.

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Table 1

F VIII Region	Primer		
Bcl I	8.1	5-	TAA AAG CTT TAA ATG GTC TAG GC -3
(Intron 18)	8.2	5-	TTC GAA TTC TGA AAT CTT GTT C - 3
Xba I	7.1	5-	CAC GAC CTC TCC ATC TGA ACA TC -3
(Intron 22)	7.10	5-	GGG CTG CAG GGG GGG GGG ACA - 3

Table 2 Clinical Severity and polymorphic status

Factor VIII units/dl	Clinical status	incidence of cases (%)	Number of cases	
			informative for Bcl I	informative for Xba I
< 2	Severe	53	6	2
2 - 10	moderately severe	26.6	3	2
>10 - 30	mild	26.6	1	1

Fig 1: Detection of Bcl I polymorphism : A 142 bp DNA fragment from intron 18 was digested with restriction enzyme Bcl I and analysed by electrophoresis using 8 % polyacrylamide gel. Lane 1 PhiX DNA/ HaeIII digest, DNA molecular weight marker. Lanes 2 & 3 show carrier and normal females & the approximate sizes of fragments are 99 & 43 bp in normal females and in carriers it is 142bp (mutant chromosome) and 99 and 43 bp (from normal chromosome). Lane 4 shows patient's sample with 142 bp (mutant chromosome).

Fig 2: Detection of Xba I polymorphism. A 96 bp DNA fragment from intron 22 was digested with restriction enzyme Xba I and analysed by electrophoresis using 12 % polyacrylamide gel. Lane 1 shows a 96 band from normal male and lanes 2,3 and 5 show DNA samples from carriers showing 96,68 and 28 bp bands. Lane 4 represents patient's sample with 68 and 28 and a 96 bp band. The extra 96 bp band which is found in all the samples is from a homologous region present elsewhere in chromosome. Lane 6, PhiX DNA /Hae III digest, DNA molecular weight marker.

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